Identifying Novel Fibrin-Binding Antibodies

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ABSTRACT

Atherosclerosis causes coronary heart disease, the leading cause of death in America today. Fibrin plays an important role in blood coagulation, especially in connection to atherosclerosis. Given the subtle and progressive nature of the disease, current diagnostic techniques are severely limited and rely on conjecture to locate plaque lesions. These shortcomings present the opportunity for the development of more specific technology. To that end, excess fibrin deposition may be exploited as a characteristic of atherosclerosis. We propose to develop an *in-vivo* fibrin-based targeting system. The first step was to select a targeting scheme that will preferentially bind to fibrin. Therefore, the Tomlinson I scFv phagemid library was used to select antibody fragments that preferentially bound a fibrin clot substrate. Three rounds of biopanning assays were performed on a fibrin clot in order to generate an enriched phage population that exhibits preferential binding to fibrin. Gel electrophoresis and DNA sequencing was used to analyze PCR samples after each screen to ensure the maintenance of the genetic insert encoding for the antibody linked to the phage coat protein and variability in the five diversity sites on the variable light and heavy chains of the displayed antibodies. After three rounds of selection, an enriched antibody population with maintained genetic diversity that exhibits preferential binding to fibrin was obtained. This is a crucial step in the identification and characterization of single antibody clones with desired binding characteristics. Future studies will involve ELISAs to identify the antibodies that most strongly bind to fibrin, SPR and immunolabeling to characterize antibody binding. It is the hope that this study and those following will progress toward the development a fibrin-based targeting system for minimally-invasive imaging modalities or delivery of therapeutics to treat atherosclerotic patients who do not exhibit symptoms of plaque formation.

INTRODUCTION AND LITERATURE REVIEW

Atherosclerosis contributes to the leading cause of death in America today, cardiovascular disease [1]. Atherosclerosis is the process by which deposits of fat, cholesterol, cellular waste products, calcium and other substances accumulate to form a plaque lesion encased by a fibrous cap in the inner lining of an artery [2]. This build-up is the result of years of exposure to stress on the vessel wall from noxious or irritating factors like smoking, hypertension, and toxins.

Atherosclerosis is a progressive disease beginning as chronic inflammation of the vascular endothelium caused by ongoing stress. The immunological component of the inflammatory response stimulates monocytes, a type of leukocyte, to migrate into the vascular tissue and differentiate into macrophages. These macrophages subsequently engulf lipids and become foam cells that form the lipid core of a plaque. Eventual endothelial erosion or plaque rupture triggers thrombosis (blood coagulation) on the surface of the lesion. Furthermore, a dislodgement of this clot may then cause embolitic events like pulmonary embolism or stroke[2].

Fibrin plays an important role in blood coagulation, especially in connection to atherosclerosis. Hemostasis, the cessation of blood loss from damaged tissue, relies on the process of blood coagulation. Platelet adhesion and aggregation and fibrin formation lead to clot formation, is the basis of blood coagulation. These factors are further characterized by Virchow's triad, which describes the three categories of factors that contribute to thrombosis. These categories are endothelial damage and injury, hemodynamic changes like turbulence or other changes in blood flow, and altered blood composition effecting properties like coagulability[3]. Arterial wall damage and impairment of blood flow due to the presence of arterial plaque lesions are the most prominent factors of the triad that cause blood coagulation in atherosclerosis.

Damage to vessel walls starts the coagulation cascade by exposing subendothelium proteins that recruit clotting factors like Factor XIII and collagen. Circulating platelets activate when they bind to collagen. In the final common pathway, thrombin cleaves the principal protein involved in coagulation, fibrinogen, to form fibrin, the building block of a hemostatic plug. As the clot forms, factor XIII crosslinks the fibrin molecules within the matrix. It is important to note that with an increase in atherosclerotic lesion complexity, there is an increase in the amount of fibrin present in the lesion[4].

Given the subtle and progressive nature of the disease, current diagnostic techniques are severely limited. The most commonly used modality to image the lumen of a vessel is X-ray angiography[5]. While it is effective in identifying lesions in a symptomatic patient, it fails to detect lesions that do not protrude into the vessel lumen. Even techniques like intravascular ultrasound and intravascular thermography rely on conjecture to locate plaque lesions. These shortcomings present the opportunity for the development of more specific technology. To that end, excess fibrin deposition can be exploited as a characteristic unique to atherosclerosis[6].

We propose to develop a fibrin-based targeting system for minimally-invasive imaging modalities or delivery of therapeutics to screen or treat pre-atherosclerotic patients who don't exhibit symptoms of plaque formation. The first step in that development is to select monoclonal antibody fragments that preferentially bind to fibrin through phage display. In 2004, Yan et al. performed a similar study, completing three rounds of screening for fibrin using the Tomlinson I and J scFv libraries. The group identified two unique antibodies[7]. It should be noted however, that screening against affinity for fibrinogen was not conducted. Fibrinogen is ubiquitous in the cardiovascular system, and given its structural similarities to fibrin, it offers a realistic competitive binding target for fibrin-specific antibodies. This study aims to preferentially select

antibody fragments that display specificity and higher affinity for fibrin over fibrinogen. To do so, soluble fibrinogen will be introduced into the screening environment in a method adapted from and imaging study conducted by Overoye et al[8], and antibodies that competitively bind to fibrinogen will be removed. Screens will be conducted using the Tomlinson I scFv phage library. We expect to identify at least one novel antibody fragment that exhibits specificity for fibrin and not fibrinogen.

Phage display is a crucial tool for the selection monoclonal antibody fragments that exhibit the specific affinities desired in this study. It was initially developed as a method for selecting peptide-displaying filamentous phage with specific binding characteristics[9]. The peptides to be displayed are expressed as fusions with the phage coat-protein pIII. During the phage assembly process, both the fusion proteins and their genetic information are packaged inside the phage particle in the form of a single-stranded DNA molecule. This physical linkage of the displayed molecule's phenotype to its corresponding genotype is the principle behind all phage display technology[10].

Phage display systems are divided into two groups based on the vector system used for the production of the phages. The library of displayed peptides is either cloned as a fusion with the phage coat protein originally in the phage genome or inserted as a fusion gene with an additional copy of the coat protein. The second production method uses phagemid vectors to produce the fusion coat-protein and yields phages that present both the wild type (original version of the coat protein) and the fusion coat protein on the same phage particle. A phagemid is a plasmid that encodes a plasmid origin of replication in addition to its phage-derived origin of replication. It lacks genetic code for proteins needed for phage assembly. This is in contrast to phage vectors that are derived from the genome of the phage and encode all the proteins needed

for the replication and assembly of the phage. Therefore the production of phages containing the phagemid genome requires additional phage-derived proteins to be present. Infecting phagemid-carrying cells with a helper phage provides the missing proteins required for replication, DNA production and packaging, and the structural proteins that form the phage coat[10].

Phagemid systems display the wild type and fusion coat protein at a certain ratio. During the phage assembly process, the wild type coat protein pIII is preferentially incorporated into the phage structure. Most phages therefore exhibit the wild type phenotype. Engineering the helper phage is one way in which the production of the wild type can be avoided. These modified helper phages, like the KM13 phage, provide all the necessary proteins for packaging the phagemid, except those of the wild type coat protein. Therefore the only coat protein that phage particles display when this modified helper phage is used is the fusion coat protein encoded on the phagemid[10].

Although phage display was initially limited to the selection of peptides, it was later shown that antibody fragments could also be successfully displayed on phage using phagemid technology[9]. While the expression levels of full-length antibodies in bacteria are generally poor, antibody fragments can be expressed at high levels in *E. coli* bacteria. This allows the selection of antibody fragments, a single polypeptide with a variable heavy (VH) and a variable light (VL) region joined together by a linker, through phage display. The most common format for the display of antibody fragments on the phage is the fusion of variable chains to the terminal pIII coat protein, as with peptide phage display systems. The Single Chain Fv antibody (scFv) library used in this study falls into the category of small antibody fragment libraries, and as it is phagemid-based, it requires the used of a helper phage.

The library of phages that present a binding antibody is enriched through a screening process that involves affinity selection on the target molecules. After each round of screening, non-binders are washed away and the antibody-displaying phage bound to the target molecule are eluted and amplified by infection into TG1 *E. coli* cells. The process is repeated after bacterial amplification of the phages selected from the previous round of screening. After completion of screening, the monoclonal antibody fragments can be produced without attachment to their phage counterparts, and can be further screened for binding to the target molecule through ELISA.

Given that current diagnostic techniques are limited based on the subtle nature of atherosclerosis, we propose to develop the basis for a fibrin-based targeting system for medical diagnostic and imaging applications. We aim to use the Tomlinson I scFv phage library and phage display technology to select for antibody fragments that preferentially bind to fibrin in a fibrinogen-rich environment, as such is found in the human body. We expect to identify at least one novel antibody that exhibits specificity for fibrin and not fibrinogen.

MATERIALS AND METHODS

Growth and Purification of Phage Libraries

(Appendix C: Procedure 1 and 2)

Frozen stock antibody libraries (1mL aliquot) were thawed on ice, diluted in 2xTY medium supplemented with 4% (wt/vol) glucose and 100μg/mL ampicillin, and grown at 37°C and 250 rpm in a 1-liter glass flask for 1.5 hours. $2x10^{11}$ CFU KM13 helper phages were added to the 50mL of culture solution to induce production of antibody-displaying phage and then incubated in a 37°C water bath for 45 minutes. Culture solutions were divided between 2 centrifuge tubes and centrifuged at 3,200g for 10 minutes at 4°C. Cell pellets were resuspended in 100mL (total) of 2xTY medium supplemented with 0.1% (wt/vol) glucose, 100μg/mL of ampicillin and 50μg/mL of kanamycin. Cultures were grown overnight (16–20 hours) at 25°C and 250 rpm in a 1-liter glass flask. Amplified phage were purified through standard PEG/NaCl purification protocol.

Titering of Produced Phage

(Appendix C: Procedure 3)

The quantity of infectious purified phage was determined through titering. *E. coli* cells were infected with serially diluted phage, spotted on tryptone/yeast extract plates supplemented with ampicillin and glucose (TAG plates), and grown overnight. After growth, resulting bacterial colonies were counted and the colony-forming units per milliliter of phage was calculated.

Selection of Antibodies

(Appendix C: Procedures 4 and 2)

The Tomlinson I Single Chain Fv antibody (scFv) phage library underwent rounds of screening with increased rigor to select for antibodies with desirable binding characteristics. A polymerized fibrin clot (2 mg/mL fibrinogen, 1 U/mL thrombin) in TBS containing the crosslinker Factor XIII (1 U/mL) and calcium chloride (500 mM) formed the screening substrate. The screening substrate and materials were blocked with MPBS to prevent non-specific binding of antibodies. Selection for antibodies with affinity for fibrin over fibrinogen were performed by incubating the phage library ($5x10^{12}$ CFU) on the polymerized fibrin clot and competitively binding off antibodies with high affinity for fibrinogen through the introduction of soluble fibrinogen (0.5 mg/mL) into the screen environment. The remaining bound phage were eluted with trypsin, amplified, and subjected to further screens. After screens, the collected phage were titered again to quantify the distribution of phage over each of the steps in the screen.

Polymerase Chain Reaction Analysis

(Appendix C: Procedures 5, 6, 7, 8, and 9)

Polymerase chain reaction of infected bacterial colonies was conducted for gel electrophoresis and sequencing analysis of the resulting phage after each round of screening; forward primer LMB3 (5'- CAG GAA AC GCT ATG AC – 3')[11]. PCR samples were analyzed for size characteristics using gel electrophoresis and ultraviolet imaging. PCR samples were also purified with QIAquick miniprep kit and submitted for sequencing by Operon. The genetic sequences were then translated to their amino acid sequence (A plasmid Editor Software; ApE) and examined for the presence of and variability in the gene insert.

RESULTS AND DISCUSSION

The aim of this study was to generate and characterize scFvs that show preferential binding to human fibrin in a competitive environment which may be used in the development of a fibrin-based targeting system for medical diagnostic and imaging applications. Screens were conducted against fibrin clots using the Tomlinson I scFv phage library.

Prior to conducting the each screen, the amplified and purified phage was titered to determine its concentration, the number of colony-forming units (CFU) and to discern the volume to be applied in screening. After each screen, the collected supernatants, washes, and elutions were titered to determine the distribution of phage bound and released at each step of the screen. The number of phage released in each step of the screen is displayed in Figure 1.

It is generally expected that since the population of fibrin-binding phage is being enriched throughout the screen iterations that the largest amount of phage would be eluted in the third screen. It is also expected that the amount of phage removed with rinses and the amount of phage that preferentially bind to fibrinogen would decrease as the population of fibrin-binding phage is enriched. In this study however, the stringency of the rinses were increased between the first and second screens, thereby influencing the number of phage removed from the screen environment. Although an overall increase in the percentage of phage eluted was not observed between the first and second screens, as attributed to the increase in rinse stringency, an increase was observed between the second and third screens when the rinse stringency remained the same. This suggests enrichment of the fibrin-binding antibody population. Future studies will identify and characterize individual strong binders of this population.

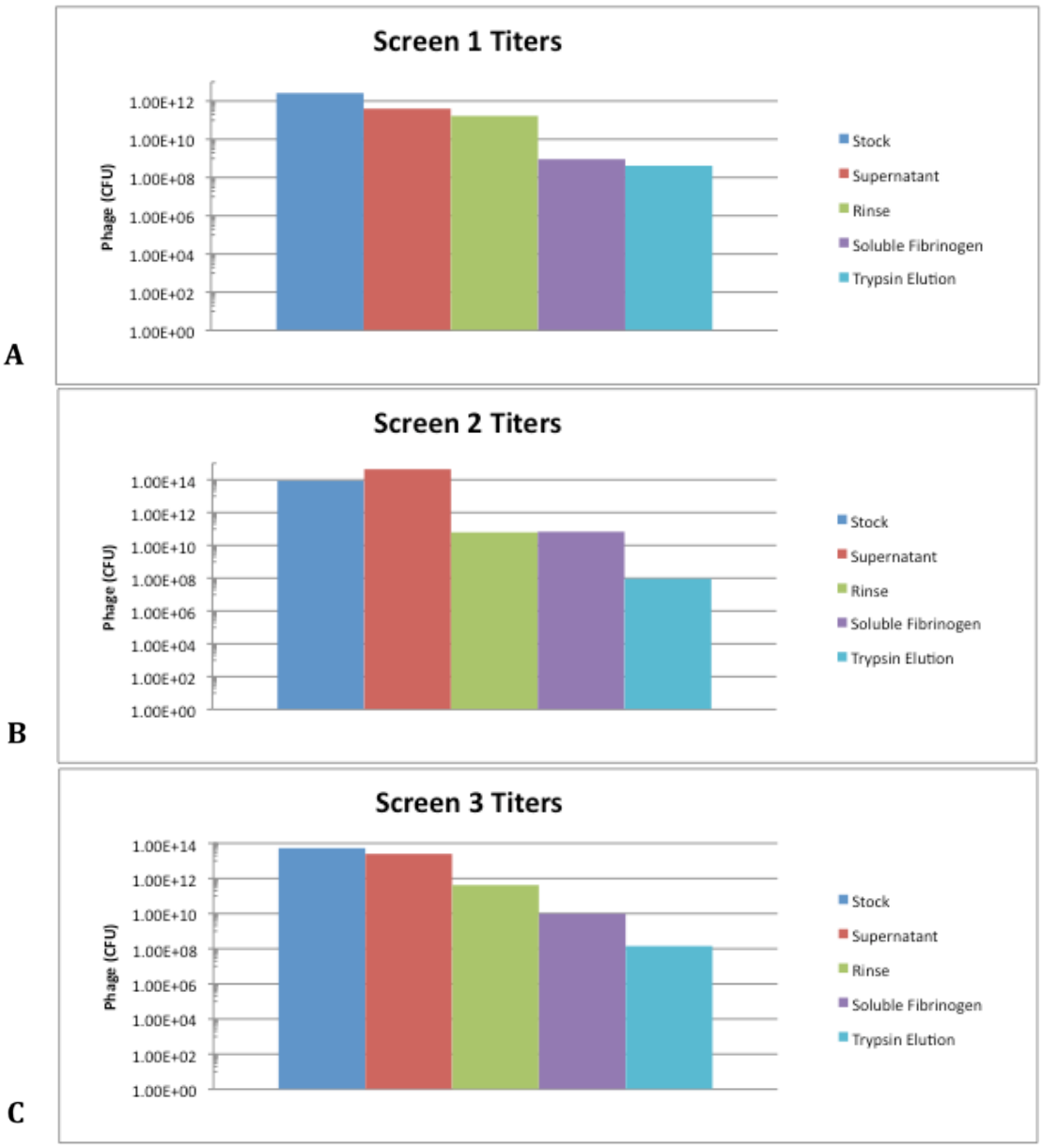
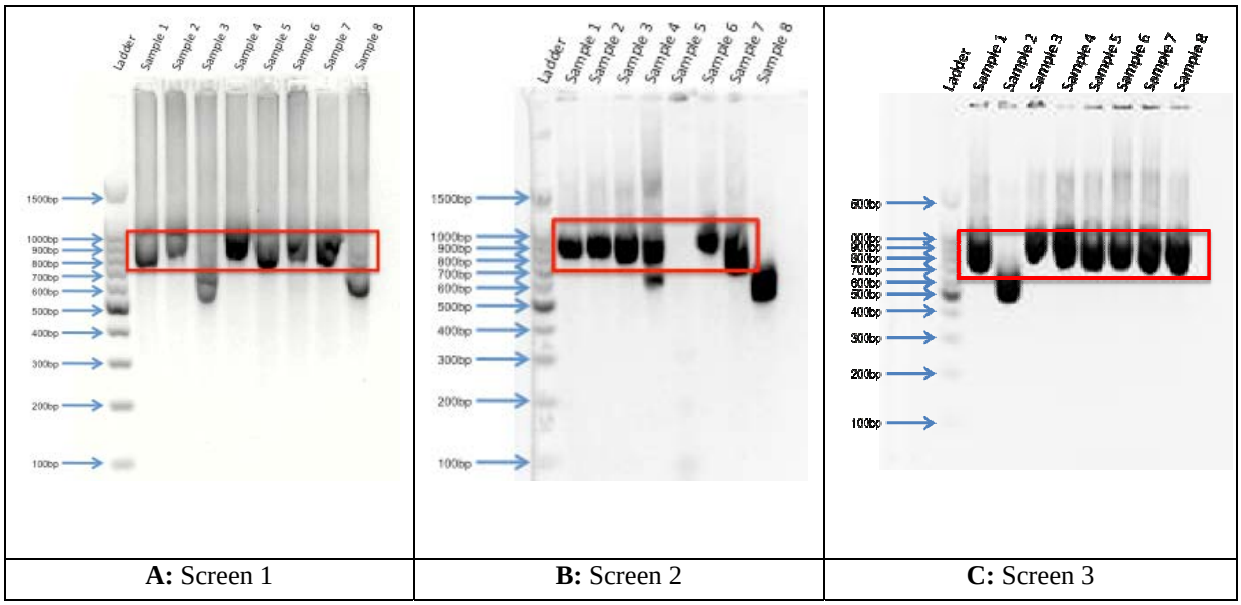


Figure 1: Screen Titers - Number of phage released (CFU) at each step in the screens (A: Screen 1; B: Screen 2; C: Screen 3) are compared on a logarithmic scale.

Gel electrophoresis analysis of PCR samples from each screen (TG1 cells infected with eluted phage from screen) had near 950 base pairs. (Figure 2) This indicates the presence of the genetic insert responsible for producing the variable antibody phenotype displayed on the phage coat protein. Genomes lacking the insert are approximately 330 base pairs.



A: Screen 1	**B:** Screen 2	**C:** Screen 3

Figure 2: Gel Electrophoresis Analysis of PCR Samples - Annotated gel images highlighting the size of PRC samples indicating maintenance of the variable antibody genetic insert

Sequencing analysis of PCR samples (Table 1) also indicates presence of the genetic insert and diversity in the four complimentary-determining regions (CDR) on the variable light and heavy chains of the displayed antibodies.

Table 1: Sequencing Analysis of PCR Samples – Deduced amino acid sequences of anti-fibrin scFvs from each screen highlighting the diversity at the CDRs on the variable heavy and light chains.

	Sample	V_H CDR2	V_H CDR3	V_L CDR2	V_L CDR3
Screen 1	1	WVSSISDAGACTT	YCAKYSSAFDYWGQGT	LLIYAASSLQSG	TYYCQQSSDDPTTFGQGT
	2	WVSSIDGTGNNTS	YCAKSSTAFDYWGQGT	LLIYXASYLQSG	TYYCQQTXXXPTTFGQGT
Screen 2	1	WVSTINYSGSSTS	YCAKGSCAFDYWGQGT	LLIYNASALQSG	TYYCQQSSSTPYTFGQGT
	2	WVSTIAGSGTATT	YCAKSSTGFDYWGQGT	LLIYAASSLQSG	TYYCQQTCYNPSTFGQGT
Screen 3	1	WVSTIASSGSATS	YCAKATTDDFTWGQGT	LLIYTASTLQSG	TYYCQQDYAGPCTFGQGT
	2	WVSSINGGGGGTG	YCAKSTTTDFTWGQGT	LLIYSASSLQSG	TYYCQQSYNDPATFGQGT

After three rounds of selection, an enriched antibody population with maintained genetic diversity that exhibits preferential binding to fibrin was created. This is a crucial step toward the identification of single fibrin-binding antibody clones. In the future, an enzyme-linked immunosorbet assay (ELISA) to be conducted on the isolated antibody-displaying phage and their soluble antibody fragments to identify the antibodies that most strongly bind to fibrin. Both forms of the antibody (conjugated to phage and independent) will be tested because an antibody may exhibit enhanced binding while conjugated to the phage body. Identified scFvs will be

sequenced to determine the number of unique clones. Surface plasmon resonance (SPR) with purified antibody fragments will be conducted to determine dislocation constants and confirm the binding specificity implied by the ELISA results. Antibody binding affinity will be further examined though the comparison of fibrin immunostaining and purified antibody-quantum dot coupled immunolabeling of atherosclerotic tissue samples. These additional studies will serve to characterize the binding affinity of the identified antibody fragments and determine the plausibility of the use of a coupled therapeutic/contrast agent-antibody targeting system *in vivo*.

CONCLUSIONS

The linkage between antibody genotype and phenotype allowed an enriched phage population showing preferential binding to fibrin to be created through the selection of monoclonal antibodies through phage display. This crucial step, together with additional studies including ELISA, SPR, and immunolabeling will serve to characterize the binding affinity of the identified antibody fragments and determine the plausibility of the use of an antibody targeting system *in vivo*. As atherosclerosis contributes to the leading cause of death in America today, it is the hope that this study and those following will progress toward the development a fibrin-based targeting system for minimally-invasive imaging modalities or delivery of therapeutics of pre-atherosclerotic patients.

APPENDIX A: REAGENTS

- Tomlinson I Single Chain Fv antibody (scFv) phage libraries, *E. coli* TGI TR strain, positive control clone (b-galactosidase-specific), negative control clone (phagemid) and KM13 helper phage
- TYE (tryptone yeast extract) agar plates and 2xTY medium (see Appendix B: Reagent Setup)
- Ampicillin solution (Sigma, catalog # A9518; see Appendix B: Reagent Setup)
- Kanamycin solution (Sigma, catalog #K1377; see Appendix B: Reagent Setup)
- Glycine solution (see Appendix B: Reagent Setup)
- Trypsin solution (Sigma, catalog #T1426; see Appendix B: Reagent Setup)
- Glucose solution (see Appendix B: Reagent Setup)
- PBS, PBST, and MT (see Appendix B: Reagent Setup)
- PEG solution (Sigma, catalog #81260; see Appendix B: Reagent Setup)
- Tween-20
- Marvel Milk Powder
- Anti-M13 horseradish peroxidase (HRP) conjugate (Amersham, catalog #27-9421-01)
- N-hydroxysuccinimide (NHS)-biotin solution (Sigma, catalog # H1759; see Appendix B: Reagent Setup)
- Sulfuric acid (see Appendix B: Reagent Setup)
- Control antigen (b-galactosidase biotinylated; Sigma, catalog # G5025)

APPENDIX B: REAGENT SETUP

- **TYE ampicillin glucose (TAG) agar plates:** Dissolve 15g of agar, 8 g of NaCl, 10g of bacto-tryptone, and 5g of yeast extract in 800mL deionized water. Autoclave. Cool down to 50 °C and add 1ml of ampicillin solution and 200mL of glucose solution. Pour plates. Plates can be stored at 4°C for up to four weeks. Plates should be dried before use.
- **TYE agar plates:** Dissolve 15g agar, 8g NaCl, 10g bacto-tryptone, and 5g of yeast extract in 1000mL deionized water. Autoclave. Cool down to 50 °C. Pour plates. Plates can be stored at 4°C for up to four weeks. Plates should be dried before use.
- **2xTY medium:** Dissolve 16g bacto-tryptone, 10g yeast extract, and 5g of NaCl in 1000mL of deionized water. Autoclave. Cool to room temperature (25 °C). Medium can be stored at RT or at 4 °C. Add antibiotic solutions and glucose solution as required and needed.
- **Kanamycin solution:** Dissolve kanamycin powder at 50mg/mL in deionized water. Filter through 0.2mM filter. Aliquot 1mL portions. Solution can be stored at -20 °C indefinitely. Thawed aliquots should be freshly diluted 1,000-fold into medium or agar.
- **Ampicillin solution:** Dissolve ampicillin powder at 100mg/mL in deionized water. Filter through 0.2mM filter. Aliquot 1mL portions. Solution can be stored at -20 °C indefinitely. Thawed aliquots should be freshly diluted 1,000-fold into medium or agar.
- **PBS buffer (1x phosphate buffer, pH 7.4):** Dissolve 3.6g of Na_2HPO_4, 0.2g of KCl, 0.24g of KH_2PO_4, and 8g of NaCl in 1000mL of deionized water. Adjust pH to 7.4 and autoclave.
- **PBST buffer:** Add 0.1% Tween-20 to PBS buffer.
- **MPSB buffer:** PBS buffer supplemented with 5% (wt/vol) marvel milk powder. Use directly and discard remaining buffer. Do not store.

- **TBS buffer (10mM Tris pH 7.4, 137mM NaCl, 1mM $CaCl_2$):** Dissolve 1.5g of Trizma base, 8g of NaCl, and 0.15g of $CaCl_2$ in 1000mL of deionized water. Adjust pH to 7.4 and autoclave.
- **PEG solution (25% PEG, 3.3M NaCl):** Dissolve 100g PEG-6000 and 73g NaCl in 500mL deionized water. Filter through 0.2µm filter. Can be stored at room temperature for up to a year.
- **Trypsin solution:** Dissolve trypsin powder at 10mg/mL in TBS (trypsin stock). Freeze in 100µL aliquots. Can be stored at -20°C for several months. For the experiment, dissolve 100µL of trypsin in 10mL TBS (trypsin solution).
- **Glucose solution:** Dissolve 200g glucose in 1000mL deionized water. Filter through 0.2µm filter. Can be stored at 4°C for several months.

APPENDIX C: PROCEDURES

1. Growth of Phage Antibody Repertoire

Duration: 1 day

1. Thaw 1mL aliquot of frozen antibody library on ice.
2. Dilute aliquot with 200mL 2xTY medium supplemented with 4% (wt/vol) glucose and 100µg/mL of ampicillin.
    - NOTE: The presence of 4% glucose allows the effective suppression of antibody expression during bacterial growth.
    - NOTE: The measurement of optical density at 600nm (OD600) in a UV-visible spectrophotometer should be 0.1. The OD600 Nanodrop readings of cultures in media containing glucose are inconsistent; therefore cultures are grown for a specified time.
3. Grow culture at 37°C and 250 rpm in a 1-liter glass flask for 1.5 hours.
4. Add $2x10^{11}$ KM13 helper phages (stored in -80°C freezer) to 50mL of culture.
5. Incubate in a water bath at 37°C for 45 minutes.
6. Divide culture/helper phage mixture between two 50mL Falcon centrifuge tubes.
7. Spin culture-containing centrifuge tubes at 3,200g for 10 minutes at 4°C.
8. Discard supernatant.
9. Resuspend pellets in 100mL (total) of 2xTY medium supplemented with 0.1% (wt/vol) glucose, 100µg/mL of ampicillin and 50µg/mL of kanamycin.
10. Grow overnight (16–20 hours) at 25°C and 250 rpm in a 1-liter glass flask.

2. Phage Purification via PEG/NaCl

Duration: 2 days

1. Spin down overnight cultures at 10,800g for 15 minutes.
2. Collect supernatant and add 15% by volume PEG/NaCl (25%PEG, 3.3M NaCl) solution.
3. Invert tubes ~50 times to mix well.
4. Incubate at 4°C for minimum 2 hours. (Can be overnight.)
5. Spin cultures at 6,000g for 45 minutes.
6. Discard supernatant and resuspend pellet in 15mL PBS.
7. Incubate phage pellet with PBS at 4°C for 30 minutes-1 hour to fully resuspend.
8. Add 15% PEG/NaCl (25%PEG, 3.3M NaCl) solution, invert 50 times
9. Incubate overnight at 4°C.
10. Spin at overnight incubations at 6,000g for 45 minutes.
11. Discard supernatant and resuspend in a minimal volume (5mL max) of PBS/BSA/EDTA (0.005M EDTA, 0.1 mg/mL BSA in PBS).
12. Incubate phage pellet with PBS/BSA/EDTA at 4°C for 30 minutes – 1 hour to resuspend.
13. Once pellet is fully resuspended, clear spin at 10,800g for 10 minutes to get rid of any residual cell debris.
14. Store supernatant at 4°C. Use phage within 1 week.

3. Phage Titering

Duration: 2 days

1. Put desired number of TAG plates in incubator to dry.
2. Inoculate 5mL 2xTY with one TGI *E. coli* colony.
3. Grow in a 15mL loose-top culture tube at 37°C and 250 rpm until the OD600 = .5.
4. Continue to grow the culture at 37°C and 150 rpm for an additional 10 minutes.
5. Dilute cells 10-fold (100µL cells in 900µL 2xTY).
6. Refrigerate cells until use.
7. Serially dilute phage to desired extent.
8. Add 10µL of phage dilution to 90µL of cell dilution in a 500µL microcentrifuge tube.
9. Incubate tubes in a water bath at 37°C for 30 minutes.
10. Label plates, and spot 15µL of infected cells in triplicate per dilution.
11. Spot 15µL controls in triplicate.
12. Incubate overnight at 30°C.
13. Count colonies and calculate average phage concentration in colony-forming units (CFU/mL) according to the following equation and compare between dilutions.

$$\text{CFU/mL} = \frac{\text{average colony count}}{.015*\text{dilution factor}}$$

4. Selection Rounds

Duration: 2 days

- NOTE: The first screen is the enrichment of antigen-specific phage by binding to immobilized antigen. The first round of selection is the most important one, as any bias or loss of diversity will be amplified in the subsequent rounds.

1. The night before screen, prep 5mL culture of starved TG1 *E. coli* cells by incubating 1 colony in 5mL of 2xTY overnight at 37°C and 250 rpm.
2. Dilute 1mL of overnight culture 100-fold in 100mL of 2xTY.
3. Grow at 37°C and 250rpm for 1.5 – 2 hours until OD600 = 0.5.
4. Refrigerate cells until eluted phage are ready for infection.
5. Set at least 6 TAG plates in the incubator to dry.

4 mg/mL Fibrinogen Solution (125µL)
26.13 µL FIB3 (@19.13 mg/mL)
62.25µL 2xTBS
1.25µL $CaCl_2$ (@500mM)
35.12µL dH_2O

2 U/mL Thrombin + FXIIIa (125µL)
2.5µL Thrombin (@100U/mL)
2.5µL FXIIIA (@100 U/mL)
62.25µL 2xTBS
1.25µL $CaCl_2$ (@500mM)
56.25µL dH_2O

6. Polymerize a 250µL fibrin gel (2mg/mL fibrinogen, 1U/mL thrombin, 1U/mL FXIIIa, 500mM $CaCl_2$ in TBS) in a 2mL microcentrifuge tube for 1 hour. See tables below for mixing proportions.
7. Block the substrate with a full volume of MPBS (5% wt/vol milk powder in PBS) at room temperature for 2 hours with slow rotation.

Screen Schedule

	Incubation Time		
	Total	**Start**	**End**
1. Fibrin Polymerization	1 hr		
2. Block w/MPBS	1 hr		
3. Rinse w/ PBS 3x			
4. Stock Phage	1 hr		
5. Rinse (Screen 1: PBST 5x, PBS 3x; Screens 2,3: PBST 10x, PBS 3x)			
6. Soluble fibrinogen	1 hr		
7. Rinse w/ PBS 3x			
8. Trypsin Elution	30min		

*For all incubations, place 2.0mL tube onto orbital shaker (tape down to inside of an old pipette tip box) to ensure even mixing of the solution.

*For rinses, add 1mL rinse solution, invert tube 5xs, then remove rinse solution.

*For step 5, in the first round of screens, rinse w/PBST 5xs and PBS 3xs. In rounds 2 and 3 screens, rinse w/PBST 10xs and 3xs with PBS.

*After phage incubation (step 4), remember to collect all supernatant solutions at each subsequent step including rinses for future titers.

- NOTE: The following table may be used as a screen schedule to outline the process.

8. Rinse screening substrate with PBS 3xs.
9. Add $5x10^{12}$ phages to the appropriate volume of MPBS buffer required to cover the selection substrate. Incubate the solution over the substrate at room temperature for 1 hour with slow rotation.
10. Collect supernatant in a microcentrifuge tube labeled “Supernatant.”

11. Wash substrate with PBST 5xs and PBS 3xs for the first screen and with PBST 10xs and PBS 3xs for screens 2 and 3. Collect wash solutions in a single 15mL centrifuge tube labeled "Rinse."
12. Incubate a full volume of 0.5mg/mL soluble fibrinogen in PBS on the substrate for 1 hour with slow rotation.
13. Collect supernatant in a 15mL centrifuge tube labeled "Soluble fibrinogen."
14. Rinse the substrate with PBS 3xs and collect the rinses in the "Soluble fibrinogen" tube.
15. To elute off phage, add the appropriate volume of trypsin solution (0.1mg/ml trypsin in TBS; dilute 10mg/mL stock trypsin at 1:100) to the substrate and incubate for 30 minutes with slow rotation.
16. Collect the elution in a 15mL tube labeled "Trypsin Elution."
17. Reserve 100µL of "Trypsin Elution" for titers.
18. Add the rest of the "Trypsin Elution" to 30mL of starved TG1 cultures (see above for prep).
19. Incubate in a water bath at 37°C for 1 hour.
20. Centrifuge culture at 3,200g for 5 minutes in one 50mL centrifuge tube.
21. Discard supernatant.
22. Resuspend cell pellet in 1mL of 2xTY medium.
23. Plate 166µL of the concentrated cell solution onto 6 TAG plates and spread with fire-polished glass Pasteur pipette.
24. Grow overnight at 37°C.
25. After overnight growth, add 3mL of 2xTY medium per plate and scrape cells off with a fire-polished glass pipette.
26. Mix all cell suspensions together in a 50mL centrifuge tube.

27. Inoculate 200mL of 2xTY + 4% glucose + 100µg/mL ampicillin medium with 1-2mL of scraped cells solution (OD600 ~0.1) in a 1L flask.
28. Store the concentrated cell solution by adding 100% glycerol to make end medium 85% 2xTY + 15% glycerol and divide into 1mL aliquots.
29. Freeze and store aliquots at -80°C.

5. Polymerase Chain Reaction

Duration: approximately 4 hours

1. The night before, streak out 3 TAG plates with desired bacterial stock.
2. Thaw PCR reagents (5x Green used for gel electrophoresis analysis or colorless used for sequencing analysis GoTaq buffer, $MgCl_2$ solution, PCR nucleotide mix (10mM each), GoTaq polymerase (5U/µL), forward primer: LMB3 (100pmol/µL), reverse primer: PHEN (100pmol/µL), Nuclease free dH_2O) on ice.
3. Create PCR master mix for selected number of clones based on the following proportions for 1 reaction (clone):
    - 4µL 5x Green or colorless GoTaq buffer
    - 2µL $MgCl_2$ solution
    - 0.4µL PCR nucleotide mix
    - 0.1µL GoTaq polymerase
    - 0.2µL Forward primer (LMB3)
    - 0.2 µL Reverse primer (PHEN)
    - 12.1 µL Nuclease free dH_2O
4. Distribute 19µL of master mix in each reaction tube.
5. Select single bacterial colonies with a 2mL micropipette and inoculate reaction one reaction mixture per colony by pipetting up and down quickly. Secure the reaction tube with a lid.
6. Load PCR reaction tubes into machine and select program “SARAH.”

6. Gel Electrophoresis Analysis of PCR Samples

Duration: approximately 3 hours

1. Set up gel form so that the plate is level and the ruler etch of gel plate is on the left-hand side.
2. Add 2µL of Gel Red Stain to a 50mL centrifuge tube.
3. Prep agarose gel (2% in 1xTAE buffer): boil solution in microwave 30 seconds – 2 minutes.
4. Pour 25mL of molten gel into the dye-containing tube.
5. Pour agarose/stain mixture into *level* gel plate holder.
6. Place the gel comb in the top groove.
7. Let the gel polymerize 1 hour.
8. Transfer plate/gel into electrophoresis device so that the top of the gel is positioned at the black (negative) electrode.
9. Cover gel with 1x TAE solution approximately 1/4" above the gel.
10. Load 7µL of sample or 3µL of 100bp ladder in each of 16 lanes.
11. Run gel at 60V for 90 minutes.
12. Image gel on gel imager in Bellamkonda lab.

7. ***Sequencing Preparation of PCR Samples***

Duration: 1 hour

1. Add 5 volumes of PB Buffer (from QIAquick miniprep kit) to 1 volume of PCR sample and mix.
2. Place a QIAquick spin column (purple) in a 2mL collection tube.
3. Apply the sample to the column and centrifuge at 13,000 rpm for 30-60 seconds.
4. Discard flow-though.
5. Place the column back into the same tube.
6. Add 400µL of PE Buffer to the column and centrifuge for 30-60 seconds. Repeat once.
7. Discard flow-through and place column back into the same tube.
8. Centrifuge the column an additional 1 minute.
    - NOTE: Additional buffer will not be completely removed unless the flow-through is discarded before additional centrifugation.
9. Place the column in a clean 1.5mL micro centrifuge tube.
10. To elute DNA, add 20µL of water to the column and centrifuge for 1 minute.
11. Measure concentrations on Nanodrop.
12. Select samples to be sequenced and inject each into its own Operon tube.
    - Samples must 20-40ng/µL and be at least 10µL in volume.
13. Dilute primer to 2µM in DH_2O and add 25µL minimum to a 1.5mL microcentrifuge tube.
14. Label this tube with a barcode sticker.

8. Registering PCR Samples for Sequencing

Duration: 30 minutes

1. Log on to operon.com
    - Username: barkerlab
    - Password: barkerlab
2. Under the “Order Now” tab, select “DNA Sequencing.”
3. Under the “Reaction Information” subsection, enter the barcode number on the tubes containing the PCR samples, their names, and the primer name.
4. Select “Process Reactions.”
5. Under the “Primer Information” subsection, enter the barcode number on the sticker used to identify the primer to be supplied.
    - The 5’-3’ sequence is not needed.
6. Under the “DN Template” subsection, enter the template type (PCR) and the size (950bp).
    - Do not select “Power Read” of “Prep/Purify.”
7. Select “Submit Samples” and go through the checkout process.

9. ORF Translation of Sequenced PCR Samples

Duration: 2 hours

1. Open .seq file in ApE program.
2. Under “Enzyme” tab, use “Enzyme Selector” tool to highlight SfiI, SmlI, and NotI sites.
    - If SfiI is not present, this may indicate that you do not have an insert present. Sometimes the NotI is not present due to a short or poor read.
    - Usually need to perform forward (LMB3 primer) and reverse (pHEN primer) reading to sequence whole insert.
3. Highlight sequence beginning right after the SfiI site (ATGG…) and ending about 120 nucleotides after NotI site.
4. Translate – Under “ORFs” tab, use “Translate” tool. Specify 10 AA per line and translate selection only.
5. Log sequence into I ORF Excel spreadsheet.

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Certificate of Research Option Approval

Wallace H. Coulter Department of Biomedical Engineering
College of Engineering
Georgia Institute of Technology

All information is to be typed. This form will not be signed by the Associate Chair for Undergraduate Studies in the Coulter Department until the student has turned in an electronic (PDF) copy of the approved thesis.

DATE: May 5, 2011

Student Name	Wendy Brown
GTID#	902251346
Major	Biomedical Engineering
Faculty Mentor	Thomas Barker
Thesis Title	Identifying Novel Fibrin-Binding Antibodies

A. Required Coursework*

Courses	Credit Hours	Semester & Year Completed
LCC 4701 *Undergraduate Thesis Writing*	1	Fall 2010
LCC 4702	1	Spring 2011
BMED 4699	3	Fall 2009
BMED 4698	3	Spring 2010
BMED 4699	3	Fall 2010
BMED 4699	3	Spring 2011

*Must contain a minimum of 9 hours of undergraduate research (i.e. BMED 2698, 2699, 4698, or 4699)

B. Other Requirements

None

C. Completion of Thesis

We, the below signed, hereby state our full approval of the thesis submitted by the above student in partial fulfillment of the requirements for the Research Option.

Thomas Barker — **Faculty mentor (print name)** — **Signature** — **Date** 5/5/2011

Philip Santangelo — **Faculty 2nd Thesis Reader (print name)** — **Signature** — **Date** 5/5/2011

D. School Approval

Approval to Grant Research Option Designation

Paul Benkeser — **Associate Chair for Undergraduate Studies** — **Signature** — **Date** 5/6/11
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